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ORIGINAL ARTICLE

Post-harvest regulated gene expression and splicing efficiency in storage roots of sugar beet (*Beta vulgaris* L.)

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Abstract Sixteen post-harvest upregulated genes from sugar beet comprising five novel sequences were isolated by subtractive cloning. Transcription profiles covering a period of up to 49 days after harvest under controlled storage conditions and in field clamps are reported. Post-harvest induced genes are involved in wound response, pathogen defense, dehydration stress, and detoxification of reactive oxygen species. An early induction of a cationic peroxidase indicates a response to post-harvest damage. Wound response reactions may also involve genes required for cell division such as a regulator of chromatin condensation and a precursor of the growth stimulating peptide phytohormone phytosulfokine-α. Surprisingly, also three putative non-protein coding genes were isolated. Two of these genes show intron specific and storage temperature dependent splicing of a precursor mRNA. The temperature dependent splicing of an intron containing sugar beet mRNA is also maintained in transgenic *Arabidopsis thaliana*. The storage induced genes are integrated into a model that proposes the response to several post-harvest stress conditions. Temperature regulated splicing may be a mechanism to sense seasonal temperature changes.

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Abbreviations

DEPC	Diethyl pyrocarbonate
CaMV	Cauliflower mosaic virus
EDTA	Ethylenediamine tetraacetic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RT	Reverse transcriptase
SDS	Sodium dodecyl sulfate

Introduction

Beta vulgaris (L.) is an important crop plant for sugar production. During the first year, the biennial plant develops an expanding storage root that accumulates sucrose in its parenchyma cells. In the second year, after vernalization during winter months, sucrose is utilized for shoot, flower, and seed production (Elliott and Weston 1993). At the end of the first year when sugar content reaches a maximum, the storage roots are harvested and stored for several months before sucrose is extracted. After harvest, the sucrose content of the storage root decreases (Jaggard et al. 1997). Most of the sucrose loss is respiratorial. The amount of energy required for the maintenance of metabolism depends on the mode of harvest and on storage temperature. Sucrose loss decreases by reducing harvest associated bruising (Wiltshire and Cobb 2000). During sucrose mobilization non-reducing sugars like glucose and fructose accumulate and contribute to the lower quality of storage roots.

Enzymes involved in sucrose mobilization in the storage root are well characterized. The sugar beet sucrose synthase

gene SBSS1 that generates UDP-glucose and fructose is expressed in the taproot under normal growth conditions and is upregulated after cold treatment and anaerobiosis in leaf and root tissue (Hesse and Willmitzer 1996). Wounding results in decreased transcription. The recently isolated second sucrose synthase gene SBSS2 shows elevated transcript levels in response to wounding, cold and short exposures to anaerobiosis (Haagenson et al. 2006; Klotz and Haagenson 2008). Interestingly, both genes may also be regulated post-transcriptionally because enzyme activity was relatively unchanged in stressed roots.

The second class of sucrose metabolising enzymes are invertases that catalyze the irreversible conversion of sucrose into glucose and fructose. There are three classes of invertases, vacuolar, cell wall bound, and neutral invertases which are located in the vacuole, apoplast, and cytoplasm, respectively (Roitsch and Gonzalez 2004). Cytosol localized neutral invertases serve catabolic purposes while cell wall bound invertases are involved in sucrose partitioning and signal transduction. Vacuolar invertases play an important role in sucrose storage and osmoregulation (Roitsch and Gonzalez 2004). Sugar beet has two cell wall bound and two vacuolar invertases (Rosenkranz et al. 2001). One of the two isoforms of each enzyme is wound inducible. Recently it was shown that both vacuolar invertases are predominantly expressed in petioles of young sugar beet plants (Gonzalez et al. 2005).

In sugar beet, one of the primary biotechnological goals is the reduction of post-harvest sugar loss as well as the decrease of impurities that affect sugar extraction (Bosemark 1993). Although the quality of the beet roots have been studied extensively during storage, little is known about post-harvest gene expression and the response to post-harvest stress conditions. Earlier gene expression studies in sugar beet concentrated on the taproot and on leaves (Bellin et al. 2002; Kloos et al. 2002; Stahl et al. 2004; Oltmanns et al. 2006; Bellin et al. 2007).

The objective of this study was the isolation of post-harvest upregulated genes, to analyse their expression profile during storage under different conditions, and to correlate gene function and gene expression with post-harvest stress responses. Surprisingly, post-harvest regulated splicing of putative non-protein coding genes was found which may function as a temperature sensing mechanism in storage roots.

Materials and methods

Experimental design

For the isolation of post-harvest upregulated genes by suppression subtractive hybridization, sugar beets of the breeding line 9K0073 (KWS Saat AG, Einbeck, Germany) were grown in the field in 1999 in Einbeck Germany. In October 1999, beets were harvested manually by first cutting of the top of the beets carrying the leaf rosette and then by pulling them from the soil. To simulate post-harvest wounding stress that beets are usually subjected to when harvested mechanically, the storage roots were treated in a cement mixer (Atika, model professional 145, Ahlen, Germany) for 1 min on the same day. Subsequently, the beets were stored in a growth chamber at either 17°C or 28°C. The 28°C temperature in one of the growth chambers was lowered to 26°C after 4 days because of dehydration of the beets. Five beets were taken from these chambers at days 1, 3, 4, 7, 14, 21, 28, 35 and 46 (17°C) and at days 1, 3, 4 (28°C), 7, and 14 (26°C) after harvest. At each time point and temperature variation a vertical slice was cut from each storage root and the slices were shredded, mixed to one sample, frozen in liquid nitrogen, and stored at −80°C.

At the day of harvest (day 0) leaf material and storage roots that were shredded but that were neither decapitated nor subjected to post-harvest wounding stress were frozen and stored at −80°C.

For the expression analysis of post-harvest upregulated genes under agronomically more comparable conditions, sugar beets of the genotype 9K0073 were grown in 1998 in the field and a clamping trial was performed in Einbeck (Germany). On 22nd October 1998, beets were harvested and approximately 30 beets were stored each in 20 nets, which were randomly distributed in a 200 t clamp of sugar beets. Samples were taken from these clamps at days 0 (the day of harvest), 1, 3, 6, 10, 14, 21, 28, 35, 42, and 49. Due to proposed differences in environmental conditions within field clamps such as temperature, moisture, and oxygen supply, six storage roots from two nets were taken from the peripheral and internal areas of the clamp. At each time point a vertical slice of 2–3 cm was cut from each of the six storage roots and combined. These slices were shredded and tissue was frozen in liquid nitrogen and stored at −80°C. The outside temperatures at the location of the field clamps was not measured directly but the temperatures were on average below 10°C during storage and at day 35 and 42 below 0°C according to local weather records.

RNA isolation and RNA gel-blot hybridizations

For RNA isolation plant material was homogenized in liquid nitrogen and resuspended in a solution containing 4 M guanidine thiocyanate, 25 mM Tris–HCl pH 8 and 100 mM β-mercaptoethanol. After centrifugation (4°C, 10 min at 3300g) nucleic acids in the supernatant were precipitated by addition of 0.03 volume sodium acetate (3 M, pH 5) and 0.75 volume ethanol (100%) and incubation over night at −20°C. After centrifugation (4°C, 10000g, 10 min) the nucleic acid

containing pellet was dissolved in 20 ml 100 mM NaCl, 10 mM EDTA pH 8, 50 mM Tris–HCl pH 8, and 0.2% SDS. Afterwards, a phenol:chloroform (1:1, v/v) and a chloroform:isoamylalcohol (24:1, v/v) extraction was performed. The pH of the aqueous solution was adjusted to about 5 with acetic acid and nucleic acids were precipitated by addition of 0.6 volume isopropanol and 0.05 volume 4 M NaCl and incubation for 2 h at −20°C. After centrifugation (20–30 min, 10000*g* at 4°C) the nucleic acid containing pellet was resuspended in 10 ml H_2O containing 0.1 % SDS. All water was treated with DEPC. Total RNA was precipitated by addition of 0.25 volume 8 M LiCl and incubation for at least 15 h at 4°C with subsequent centrifugation for 20 min at 4°C, 10000*g*. Total RNA was resuspended in 400 µl H_2O. After ethanol precipitation (addition of 0.1 volume sodium acetate, 3 M pH 4.8, and 2.5 volume ethanol) total RNA was resuspended in a volume of 50–100 µl H_2O. The isolation of poly(A) + RNA was carried out with the Oligotex Kit according to the manufacturers protocol (Qiagen, Hilden, Germany). For RNA preparation from *Arabidopsis thaliana*, the NucleoSpin® RNA Plant kit from Macherey Nagel (Düren, Germany) was used. Measurements of RNA yield, electrophoretic separation on formaldehyde gels, and RNA-blot hybridizations were done following standard protocols (Ausubel et al. 1988; Sambrook et al. 1989). For RNA-blot hybridizations 10 µg total RNA was employed. Radioactive probes were generated with gel purified DNA fragments with the HexaLabel DNA Labeling Kit (Fermentas, St Leon-Rot, Germany) or were custom made by Hartman Analytic (Braunschweig, Germany).

Suppression subtractive hybridization

The synthesis of cDNA was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech, Heidelberg, Germany). Each synthesis was carried out with 8 µg poly(A) + RNA from pre-storage taproots (day 0) or taproots that were taken from the 17°C incubation chamber between 1 and 46 days after harvest (see above). Subtractive hybridization was done following the user manual (PT1117–1) of the Clontech PCR-Select™ cDNA Subtraction Kit. Two subtractions were carried out. A forward subtraction to enrich for post-harvest induced genes that were upregulated 1–46 days after harvest and a reverse subtraction to enrich for genes expressed at day 0 before storage. For the forward subtraction equal amounts of cDNA from all time points were mixed and employed for subtraction.

After the second PCR (user manual), the amplified fragments from the forward subtraction were cloned into the PCR cloning vector pCR®2.1 (T/A Cloning Kit®; Invitrogen, Karlsruhe, Germany). Prior to ligation into pCR2.1, the subtracted PCR cDNA products were subjected to an additional incubation of 1 h at 72°C with dATP and *Taq* polymerase (TaKaRa, Gennevilliers, France) to ensure that the majority of the PCR fragments contain "A-overhangs" for an efficient cloning into the T/A cloning vector. About 200 ng of the PCR amplified cDNA was ligated to 50 ng pCR2.1. Ligations were transformed into competent *E. coli* INVαF' (Invitrogen) and recombinant clones were selected as described by the manufacturer. Recombinant clones (284) were further characterized by differential hybridization with forward- and reverse-subtracted cDNA probes as described in the user manual of the PCR-Select™ cDNA Subtraction Kit. After this differential screening, 62 clones were identified that hybridized stronger or specifically with the probe from the forward subtraction. The inserts of these plasmids were sequenced with fluorescently labeled M13 reverse and forward (−20) primers using the AutoRead Sequencing Kit (Pharmacia, Freiburg, Germany) and the Automated Laser Fluorescent ALF™ DNA Sequencer from Pharmacia LKB. DNA sequence analysis was also performed by Amodia GmbH (Braunschweig, Germany). After sequence analysis 35 different clones remained. RT-PCR analysis revealed that 16 of these clones correspond to post-harvest upregulated genes (for primers, see below).

cDNA cloning and sequencing

Poly(A) + RNA was isolated with the Oligotex™ mRNA mini/midi kits (Qiagen). For cDNA synthesis 5 µg poly(A)+ mRNA isolated from equal amounts of total RNA from storage roots kept for 3 and 46 days at 17°C after harvest was employed. cDNA synthesis and cloning was performed according to the SuperScript™ Plasmid System (Invitrogen) and 2×10^5 primary clones were obtained. The library was amplified and screened with cDNA fragments from the forward subtraction according to standard protocols (Sambrook et al. 1989). Recombinant clones harbouring the largest inserts were sequenced (Seqlab GmbH, Göttingen, Germany).

Genomic cloning

A genomic library from sugar beet genotype 1K0088 generated in the lambda vector EMBL3 SP6/T7 (Stahl et al. 2004) was used for the isolation of genomic clones for *Bvnpcg2* and *Bvnpcg3* according to standard protocols (Sambrook et al. 1989). A 13.1 kb *Xho*I fragment containing *Bvnpcg2* was isolated from a recombinant lambda clone and cloned into pBSK + (Stratagene, Amsterdam, The Netherlands). This clone was partially sequenced (Seqlab). A recombinant lambda clone for *Bvnpcg3* was partially sequenced with a PCR based approach using the BigDye Term v1.1 Cycleseq Kit from Applied Biosystems (Darmstadt, Germany) and the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems).

RACE-PCR

In one case (*Bvglp-r*), RACE-PCR was employed to obtain a complete cDNA. For RACE-PCR the Marathon™ cDNA amplification kit (Clontech) was used with the following gene specific primer: 5′-CTGATGCTCGGTGTGAGTGAATCG GAAC-3′. Poly(A) + RNA was isolated from total RNA from storage roots after 46 days at 17°C. Amplified fragments were cloned into pCR2.1 (Invitrogen) and sequenced (Seqlab).

RT-PCR

For RT-PCR analyses, first strand cDNA was generated with 5 µg total RNA with the RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas) in a final volume of 20 µl. 40 pg template cDNA in 2.5 µl was mixed with 2.5 µl 10 × PCR-buffer, 1 µl of each gene specific primer (5 pmole/µl), 0.5 µl dNTP-mix (10 mM), 17.4 µl ddH_2O, and 0.1 µl Taq-DNA polymerase (5 u/µl). Enzyme and 10 ×-buffer were obtained from Invitrogen and Qbiogene (Eschwege, Germany). PCR reactions were carried out in an Eppendorf Mastercycler (Hamburg, Germany) under the following conditions. 5 min at 94°C followed by 27 cycles at 94°C (30 s), 52°C (1 min), 72°C (1.5 min). Final incubation was at 72°C for 10 min. All RT-PCRs were carried out under the same conditions except for *Bvpsk*, on which a reamplification was performed. For this 2.5 µl from the first PCR were subjected to a second PCR under the same conditions except the cycles were reduced from 27 to 20. Gene specific primers are listed in Table 1. The primers for *Bvgapdh* and *Atgapdh* were derived from GenBank accession numbers AW697769 and NM_111283, respectively. All other primers were derived from the cDNA sequences deposited in GenBank. Accession numbers are listed in Table 2.

Computer assisted sequence analysis

The sequences of all clones were processed and analysed with the computer programmes DNA Strider and WinGene under Macintosh or Windows operating systems, respectively (Douglas 1995; Hennig 1999). For the identification of homologues, the Basic Local Alignment Search Tool (BLAST) at http://www.ncbi.nlm.nih.gov/BLAST/ was used (Altschul et al. 1997). Conserved domains were identified at the same web server (Marchler-Bauer et al. 2007). Identification of signal peptides and subcellular localization was done with the PSORT program at http://www.psort.org/ (Nakai and Kanehisa 1991). Calculation of the pI of each protein was done with ProtParam at http://www.expasy.ch/tools/protparam.html (Gasteiger et al. 2005).

Arabidopsis thaliana transformation

A cDNA for *Bvnpcg3* containing an intron was expressed in *Arabidopsis thaliana* L. cv Columbia (Nottingham Arabidopsis Stock Center, Loughborough, United Kingdom).

Table 1 Gene specific primers

Gene	Primer 1	Primer 2
Bvhsp83	5′-TTCCAAGAAGACAATGGAGATTAAC-3′	5′-AAAGGAACAACCTTAATCAACTTCC-3′
Bvcprx1	5′-GACTCCAATTCTAGCCATCTTGAC-3’	5′-AATGAAACATTCCTTGATCAAACTC-3′
Bvrip-r1	5’-AAGTTTCTTAACAAGACTCCGCAAT-3’	5′-TGAGTCCCAATACAATGTTATTCCT-3′
Bvrip-r2	5′-AAGATAGCCCTCCAAATATAAATCG-3′	5′-TGAACTTGAATCTGTTAACTGCTGA-3′
Bvb12d	5′-AATCAGCAGGCTGTGAGTGTATAAT-3′	5′-CTCCAGAGATTATGCCTTCTATCAA-3′
Bvglp-r	5′-CCAAAGATTCTCTTCAATTTCTTCA-3′	5′-GGGGATATTATGATTTTTCCACCTA-3′
Bvsp2	5′-ATGTAAGATTATCACCAGGAGCAAC-3′	5′-CTGTCATGGAACTACAACTATGCTC-3′
Bvc561r	5′-ACATGCTTCAAGTCTACAAACTAAACT-3′	5′-TTCTCCAAAGATATATGGAAGTTACG-3′
Bvbpm1	5′-CACAGAGAAAGTAACCAAAGACACC-3′	5′-TAATTTTCAATGAGGAGAAATCCTG-3′
Bvpsk	5′-AAAACCTTTGCAAAATTTAAAATCA3′	5′-TGAGAGTTGTCAAGGAGTAAAATGA-3′
Bvrcc	5′-CTTATTCTCCGTCAATGGTTTTATG-3′	5′-ACAATCTCTTGAATCTTCCTCTTCA-3′
Bvmarb	5′-ACTGTATGCTAAGGCTGTGAAATTG-3′	5′-TTGTGTTCATTCTGCTTTTCAGATA-3′
Bvwdrp	5′-TTCCTAACATAGACAAGAACATTTGC-3′	5′-AATATATGGTTATTTGGTCCCCTTT-3′
Bvnpcg1	5′-ATTGATGTGTTGTCAAGTTGTTGTT-3′	5′-AACATCATATGAAAGTTTGGCACTT-3′
Bvnpcg2 1. intron	5′-GCAGATCCAATTAACTGTTACG-3′	5′-TTCTCGAATTCTCCTTTCTCTC-3′
Bvnpcg2 2. intron	5′-AGAGACACAGACACAAAAGGG-3′	5′-AATTGGATCTGCTTGATTCG-3′
Bvnpcg2 3. intron	5′-CTTAAGTAGCTGACAAGCATCAACC-3′	5′-TTTCTTTGGAAGAGAGATTTGTTTG-3′
Bvnpcg3 2. intron	5′-ATAAAGGCTGGATGGAGAACTGTAG-3′	5′-ACGAGAAACACCCCTCTTAAGTAAT-3′
Bvgapdh	5′-ATGTTTAAGTACGACAGTGTTCACG-3′	5′-ATGTGAAGGTCTGACTTGTATTCGT-3′
Atgapdh	5′-GGAATTGTTGAGGGTCTTATGACTA-3′	5′-CTGACAGTAAGGTCAACAACTGAGA-3′

Table 2 GeneBank Accession numbers of cDNA sequences and corresponding expressed sequence tags (ESTs) from sugar beet

Gene	cDNA Accession numbers	ESTs[a] Accession numbers identity (%)	References
Bvhsp83	AM265618	BQ583628 (97)	Herwig et al. (2002)
Bvcprx1	AM265608	BQ595395 (98)	Herwig et al. (2002)
Bvrip-r1	AM265609	None	
Bvrip-r2	AM265610	None	
Bvb12d	AM265611	BF011036 (97)	
Bvglp-r	AM265612	BI643055 (81)	
Bvsp2	AAA32916	ND[b]	Nielsen et al. (1994)
Bvc561r	AM265613	BQ591851 (87)	Herwig et al. (2002)
Bvbpm1	AAB67868	ND[b]	Qi et al. (1996)
Bvrcc	AM265615	None	
Bvmarb	AM265616	BQ589682 (98)	Herwig et al. (2002)
Bvwdrp	AM265617	DV501821 (98)	
Bvnpcg1	AM265619	BQ593372 (100)[c]	Herwig et al. (2002)
Bvnpcg2	AM265620	None	
Bvnpcg3	AM265621	BQ594387 (99)	Herwig et al. (2002)
Bvpsk	AM265614	None	

[a] only the highest scoring sugar beet EST is listed with % identity

[b] *ND* not determined. ESTs for previously published genes are not listed

[c] EST shows a stretch of 100% identity within 143 nucleotides of both sequences

The T-DNA construct for *Arabidopsis* transformation was generated with the binary vector pVKH-35S-pA1 that harbors the CaMV 35S promoter and a polyA addition signal (Reintanz et al. 2002). This plasmid was cut first with *Bam*HI, the ends filled in and then cut with *Hin*dIII. This generates compatible ends to directionally clone a *Sma*I/*Hin*dIII *Bvnpcg3* cDNA fragment isolated from the recombinant pSPORT1 plasmid used for cDNA cloning. The recombinant T-DNA vector was transformed into *Agrobacterium* strain c58C1 harbouring the resident plasmid pGV2260 (Deblaere et al. 1985). Transformation of *Arabidopsis thaliana* was carried out with the floral dip method (Clough and Bent 1998). Transgenic offspring of primary transformants were subjected to RT-PCR analysis.

Results

Post-harvest upregulated genes

Using suppression subtractive hybridization (SSH) 16 differentially enriched cDNA fragments were identified that correspond to post-harvest upregulated genes. Twelve cDNAs with a complete reading frame were cloned. For the gene encoding the heat shock protein *Bvhsp83* no cDNA was isolated that encodes the entire protein. All cDNAs were designated according to the homology of the encoded protein. Three cDNAs did not encode a known protein. These were designated as putative *Beta vulgaris* non-protein coding genes (*Bvnpcg1-3*).

To investigate if the genes have been isolated from sugar beet before, a blastn comparison with GenBank sequences was performed (all non-redundant nucleotide sequences and ESTs). Table 2 lists the sixteen genes and the EMBL/GenBank accession numbers of the cloned cDNA sequences and their homologs from sugar beet. Only two of the genes, *Bvsp2*, a chitinase, and *Bvbpm1*, an aquaporin, have been described earlier (Nielsen et al. 1994; Qi et al. 1996). When the EST databases for *Beta vulgaris* were screened with the isolated 16 sequences, five genes were not detected (Table 2). The EST database contains more than 26,800 entries for *Beta vulgaris* (6 November, 2007). This indicates either a very low transcription rate or a high post-harvest expression specificity of *Bvrip-r1*, *Bvrip-r2*, *Bvrcc*, *Bvnpcg2*, and *Bvpsk*.

When the encoded amino acid sequences were analysed with the blastp programme, homologous proteins and conserved domains were identified. Table 3 shows the result of the blastp analysis and lists the name and accession number of the highest scoring protein sequence for which a function is proposed. The very low E-value obtained in the blastp analysis corresponds with a strong significance of the proposed homology. The cloned sugar beet genes encode a heat shock protein (*Bvhsp83*), a cationic peroxidase (*Bvcprx1*), two ribosome inactivating proteins (*Bvrip-r1* and *Bvrip-r2*), a B12D protein (*Bvb12d*), a germin-like protein (*Bvglp-r*), a chitinase (*Bvsp2*), a cytochrome b561 oxidase (*Bvc561r*), an aquaporin (*Bvbpm1*), a regulator of chromatin condensation (*Bvrcc*), a matrix attachment region binding protein homolog (*Bvmarb*), a protein that harbours WD40 repeats (*Bvwdrp*), and a precursor of the peptide phytohormone phytosulfokine-alpha (*Bvpsk*).

The proposed function of the cloned sugar beet genes is further supported by the conserved domain database analy-

Table 3 GeneBank accession numbers of protein sequences and corresponding homologs

Sugar beet protein		Homologues[a]				
Protein	Accession numbers	Protein	Accession numbers	Species	E-value	References
BvHSP83	CAK22426	HSP83	P51819	*Ipomoea nil*	8e-158	Felsheim and Das (1992)
BvCPRX1	CAK22416	Peroxidase	CAA71489	*Spinacia oleracea*	8e-155	Simon (1993)
BvRIP-R1	CAK22417	Ribosome-inactivating protein	BAB83507	*Spinacia oleracea*	5e-117	
BvRIP-R2	CAK22418	PAP-I (ribosome-inactivating protein)	P10297	*Phytolacca americana*	2e-38	Lin et al. (1991)
BvB12D	CAK22419	B12D protein	AAD22104	*Ipomoea batatas*	7e-32	Huang et al. (2001)
BvGLP-R	CAK22420	Germin-like protein Kiel 1	AAO92348	*Beta vulgaris*	8e-83	
BvSP2	–[b]	Chitinase	AAA32916	*Beta vulgaris*		Nielsen et al. (1994)
BvC561R	CAK22421	Cytochrome B561-related	NP_193560	*Arabidopsis thaliana*	1e-62	
BvBPM1	–[b]	Plasma membrane major intrinsic protein 1	AAB67868	*Beta vulgaris*		Qi et al. (1996)
BvRCC	CAK22423	UVB-resistance protein-related/regulator of chromosome condensation (RCC1) family protein	NP_568250	*Arabidopsis thaliana*	4e-171	
BvMARB	CAK22424	MAR-binding protein	BAB41076	*Nicotiana tabacum*	0.0	
BvWDRP	CAK22425	Transducin family protein/WD-40 repeat family protein	NP_188525	*Arabidopsis thaliana*	3e-121	
BvPSK	CAK22422	Putative phytosulfokine peptide precursor	DAA00288	*Mesembryanthemum crystallinum*	1e-14	Lorbiecke and Sauter (2002)

The genes *Bvnpcg1*, *2* and *3* are not included because they do not encode a known protein

[a] The highest scoring homologue in a blastp analysis that was associated with a putative protein function is listed

[b] Amino acid sequence is identical to the homologous protein

sis (Marchler-Bauer et al. 2007). Table 4 shows that all proteins have functional domains associated with their protein homology (Table 3), except one. BvMARB has four domains characteristic for Nop protein families of yeast. The N terminal NOP5NT domain is found in RNA-binding proteins of the NOP5 family. Nop5p is a small nucleolar ribonucleoprotein component required for pre-18 S rRNA processing in yeast (Wu et al. 1998). NOSIC is the central domain found in Nop56/SIK1-like proteins. Nop56p was identified in yeast as a component of the ribonucleoprotein particles (Venema and Tollervey 1999). The similarity of matrix attachment region binding proteins (MARBs) with the Nop5/56 proteins of yeast has been shown before and may imply that MARBs also have a function in ribosome maturation (Hatton and Gray 1999).

According to a further analysis of the amino acid sequences, the proteins BvCPRX1, BvRIP-r1, BvRIP-r2, BvGLP-r, BvSP2, and BvPSK may all harbour a transit peptide at their N-terminus (Table 5). Of these, only BvRIP-r1 is proposed to harbour a non cleavable signal peptide leading to a retention within the endoplasmatic reticulum.

Transcript profiles of post-harvest upregulated genes

To investigate post-harvest transcription, RT-PCR analyses were performed with RNA from two controlled storage experiments and with RNA derived from sugar beet field clamps. All transcripts were amplified from stored sugar beet roots (Figs. 1a, 3c; R 17°C, R 28/26°C). All but *Bvmarb*, *Bvnpcg2* and *Bvnpcg3* were more highly expressed during storage (Figs. 1a, 3c; compare R 0 with R 17°C and R 28/26°C). In four cases transcript can also be amplified from leaves (Fig. 1a; L 0: *Bvsp2, Bvpsk*. Fig. 3c, L 0: *Bvnpcg2* and *3*). Thirteen genes could also be amplified from field clamp derived storage roots (Figs. 1b, 3c: R < 10°C). Transcripts of the genes *Bvglp-r*, *Bvwdrp*, and *Bvnpcg1* were not detected under these experimental conditions (data not shown). After reamplification, transcripts of *Bvpsk* could be detected at all post-harvest time points except day 0 (Fig. 1). No reamplification was performed for *Bvglp-r*, *Bvwdrp*, and *Bvnpcg1*.

Bvcprx1, *Bvrip-r1*, *Bvsp2*, *Bvrcc*, and *Bvmarb* were amplified from almost all time points regardless under which storage conditions (Fig. 1). Other genes show a more specific amplification pattern. As expected, the transcript of the heatshock protein HSP83 was more readily detected at higher temperatures than at lower temperatures (Fig. 1a). *Bvglp-r* and *Bvb12d* are most highly expressed after 46 days of storage at 17°C while *Bvb12d* is also readily detected at higher temperatures and weekly detected at 17°C one and three days after harvest (Fig. 1a; 17°C, 28/26°C). In storage roots from field clamps, transcripts from both genes are not or hardly amplified (Fig. 1b and data not shown). *Bvrip-r2*

Table 4 Conserved domains within storage induced proteins

Protein	Length of protein (amino acids)	Domain/position	Identifier	E-value
BvHSP83	350	HSP90/1–350	pfam00183	7e-139
		Molecular chaperone, HSP90 family/1–321	COG0326	2e-84
		PRK05218, heat shock protein 90/1–316	PRK05218	2e-83
BvCPRX1	326	Secretory_peroxidase/26–322	cd00693	2e-100
		Plant_peroxidase/33–307	cd00314	9e-64
		Ascorbate_peroxidase/24–313	cd00691	2e-10
		Peroxidase/45–290	pfam00141	1e-65
BvRIP-r1	321	Ribosome-inactivating protein/39–294	pfam00161	6e-39
BvRIP-r1	290	Ribosome-inactivating protein/23–269	pfam00161	8e-43
BvB12D	91	B12D protein/4–90	pfam06522	2e-29
BvGLP-r	211	Cupin_1/56–185	pfam00190	7e-13
		Cupin_2, cupin domain/91–165	pfam07883	8e-04
		Contains double-stranded beta-helix domain/54–180	COG1917	0.001
BvC561r	264	B561, cytochrome b-561/56–180	smart00665	9e-20
		Cytochrome_B-561/21–222	pfam03188	6e-06
BvRCC	573	Regulator of chromosome condensation/31–87	pfam00415	0.008
		Alpha-tubulin suppressor and related RCC1 domain-containing proteins/1–480	COG5184	2e-32
BvMARB	572	Nop, putative snoRNA binding domain/251–399	pfam01798	1e-59
		NOSIC, (NUC001) domain/159–211	pfam08060	4e-22
		NOP5NT, (NUC127) domain/1–66	pfam08156	7e-16
		SIK1, protein implicated in ribosomal biogenesis, Nop56p homolog/42–432	COG1498	4e-94
BvWDRP	503	WD40 domain/134–492	cd00200	2e-26
		WD40 repeats/276–314	smart00320	0.003
		WD domain, G-beta repeat/277–314	pfam00400	0.004
		FOG: WD40 repeat/160–503	COG2319	3e-04
BvPSK	76	PSK, phytosulfokine precursor protein (PSK)/5–76	pfam06404	3e-11

The genes *Bvnpcg1*, *2*, and *3* are not included because they do not encode a known protein

Bvsp2 and *Bvbpm1* are not included because these genes have been published before (Table 2)

Table 5 N-terminal signal peptides identified in proteins BvCPRX1, BvRIP-r1, BvRIP-r2, BvGLP-r, BvSP2, and BvPSK

Protein	Signal peptide
BvCPRX1	MRETSFSFVL FLGLVLITLV GHCYG
BvRIP-r1	MKALTAARWI QWCIIMVVVI VPITAT
BvRIP-r2	MTNCFLVVVL AIWIVPA
BvGLP-r	MDALKIVFLF ALLVPLSSA
BvSP2	MALLLKNTLY IALIISVISS FPTSLFA
BvPSK	MSKFTTLLII VMLVCFAST

transcript was hardly detected at 1–3 days after harvest at 17°C and is only prominent at higher temperatures 1 day after harvest (Fig. 1a; 28/26°C). In general, less genes are detected to be upregulated in field clamps than under controlled storage conditions. This may be due to the lower physiological activity of the storage root at lower field clamp temperatures. When the RT-PCR amplification results of the genes isolated by subtractive cloning are compared with those of the house keeping gene *Bvgapdh* amplified under the same conditions it may be concluded that all genes except *Bvmarb* are post-harvest upregulated.

To support the conclusion that the RT-PCR experiments detect changes in quantitative gene transcription, RNA gel-blot analysis were performed on selected genes as well. The detection of the mRNA of *Bvhsp83*, *Bvcprx1* and *Bvrip-r1* in stored storage roots corresponds to the transcript profiles detected by RT-PCR (compare Fig. 2 with Fig. 1).

Intron specific splicing efficiency of putative non-protein coding genes during storage

Three of the cDNAs that were isolated do not harbour a reading frame that shows homology to a known protein.

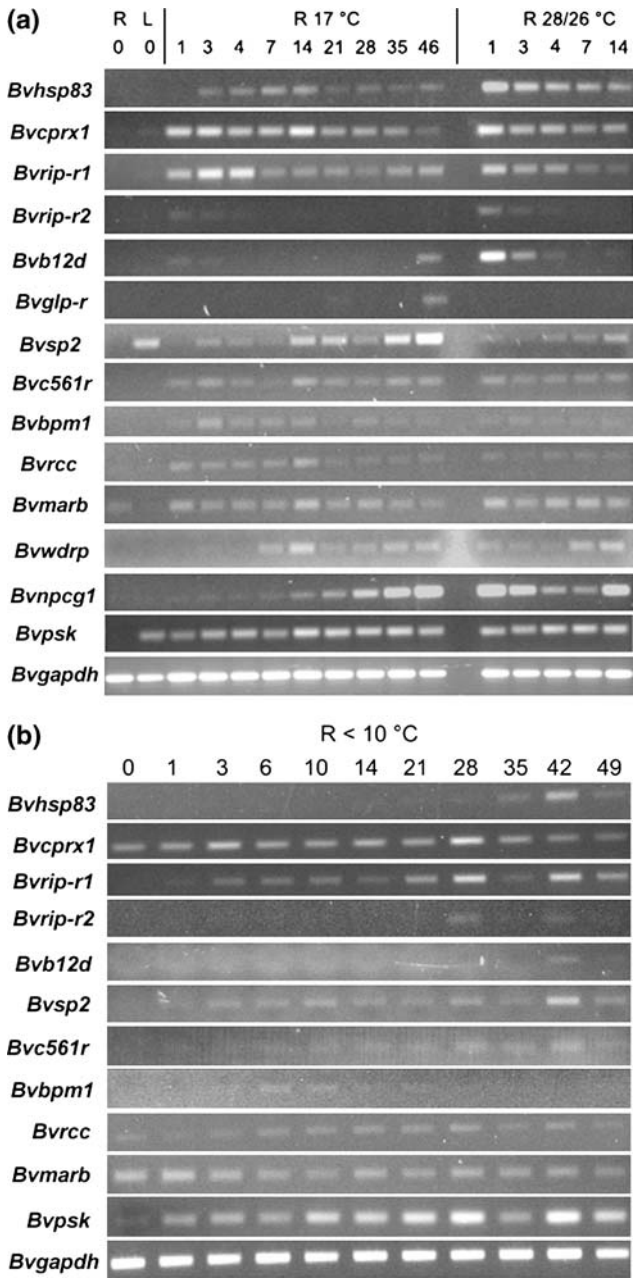

Fig. 1 Post-harvest regulated gene expression. RT-PCR analyses were performed with gene specific primers on cDNA generated from either leaves (L) or storage roots (R) mRNA of sugar beet that were kept at different temperatures (°C) and for different time periods (0–49 days). *Bvgadph*, a house keeping gene, was amplified as a control. **a** Results from temperature controlled storage experiments. Results for genes *Bvnpcg2* and *3* are shown in Fig. 3c. *Bvpsk* fragments were detected after reamplification. **b** Results from a field clamp storage experiment. Results for genes *Bvnpcg2* and *3* are shown in Fig. 3c. *Bvglp-r, Bvwdrp*, and *Bvnpcg1* were not included because no fragments were amplified with the PCR conditions used. *Bvpsk* fragments were detected after reamplification

These three genes are putative non-protein coding genes (*Bvnpcg1*, *2*, and *3*). RT-PCR experiments with primers for the cDNA fragments amplified two fragments for *Bvnpcg2*

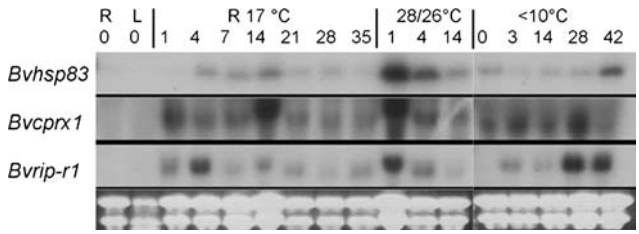

Fig. 2 Post-harvest regulated gene expression. RNA gel-blot analyses were performed with radioactively labelled cDNA fragments for *Bvhsp83*, *Bvcprx* and *Bvrip-r1* on total RNA from either leaves (L) or storage roots of sugar beet (R) that were kept at different temperatures (°C) and for different time periods (0–42 days). Ethidium bromide staining of the RNA gel is shown below

and *Bvnpcg3* (Fig. 3c). In both cases the smaller fragment corresponds in size to the PCR fragment isolated by SSH (data not shown). The larger fragment could originate from a second amplified gene or from differential splicing. To investigate this, genomic clones were isolated for *Bvnpcg2* and *Bvnpcg3*, sequenced and compared with the cDNA sequence. Comparing cDNA and genomic clones from *Bvnpcg2* revealed the presence of three introns (Fig. 3a). The third intron was located in the region amplified by RT-PCR and corresponds in size to the size difference observed for the two fragments in the RT-PCR experiment (Fig. 3a, c). The cDNA isolated for *Bvnpcg3* (Fig. 3a) harboured an intron that was absent in the cDNA fragment isolated by subtractive cloning. Sequence comparison between the genomic clone and the cDNAs confirmed the presence of this intron and showed that this was the second intron of the *Bvnpcg3* gene (Fig. 3a). This second intron was located in the region amplified by RT-PCR and corresponds in size to the size difference observed for the two fragments in the RT-PCR experiment (Fig. 3a, c). Therefore, the two fragments observed in the RT-PCR experiments are due to amplification of a spliced and unspliced mRNA of each gene. RNA gel-blot experiments revealed the presence of the second intron in the mRNA of *Bvnpcg3* while intron retention could not be detected for *Bvnpcg2* in RNA gel-blots (Fig. 3b). RNA gel-blot experiments further show that the length of the cloned cDNAs correspond in size to the hybridizing mRNA (Fig. 3b). This indicates that the cDNAs are full size or almost full size clones. The first and second intron of *Bvnpcg2* were also never detected in RT-PCR experiments with primers flanking these two introns (Fig. 3c). Furthermore, while the size of the first intron of *Bvnpcg3* may be too large to be efficiently detected by RT-PCR (>3 kb), RNA-gel blot experiments do not detect an mRNA corresponding in size with the presence of the first intron (Fig. 3b). Therefore, the observed difference in splicing efficiency is intron specific.

When the relative amount of spliced and unspliced fragment is compared under the different storage conditions, it

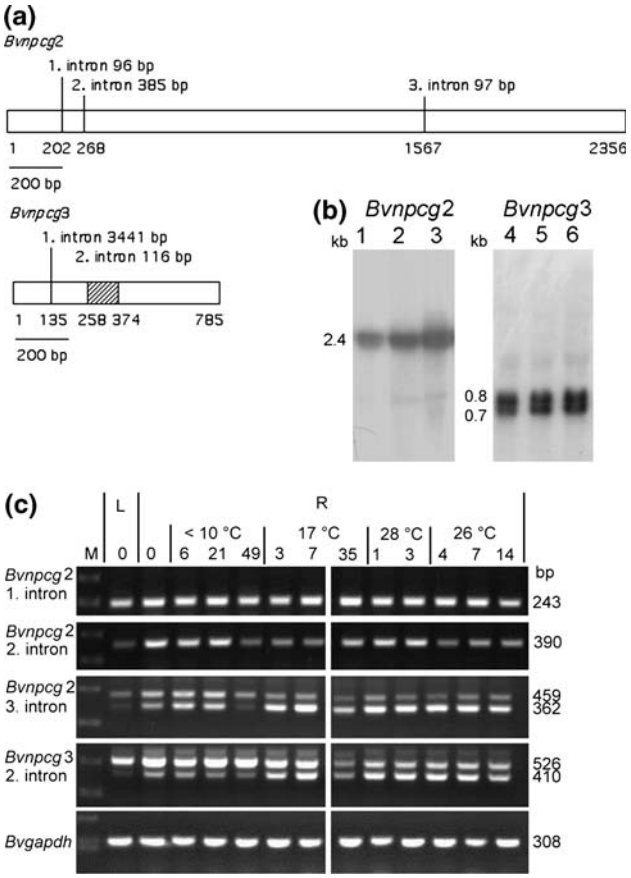

Fig. 3 Post-harvest regulated splicing of *Bvnpcg2* and *Bvnpcg3* mRNAs. *Bvgadph*, a house keeping gene, was amplified as a control. **a** Schematic representation of the two cDNAs indicating the size and position of the introns. The second intron of *Bvnpcg3* present in the cDNA is shown. **b** RNA gel-blot hybridizations with *Bvnpcg2* and *Bvnpcg3* and total RNA from taproots stored at 17°C for 4 (*lane 1*), 7 (*lane 2*) 14 (*lane 3*), 3 (*lane 4*), 7 (*lane 5*), and 28 (*lane 6*) days. The size of the hybridizing RNA is given in kb. **c** RT-PCR analyses were performed with gene specific primers bordering the three introns of *Bvnpcg2*, the second intron of *Bvnpcg3*, and primers for *Bvgapdh* on cDNA generated from either leaves (L) or storage roots (R) mRNA of sugar beet that were kept at different temperatures (°C) and for different time periods (0–49 days). The first lane (M) harbours a DNA size standard. The 500 (*top*) and 250 bp (*bottom*) fragments can be seen in the marker lane. Sizes of amplified fragments are given in bp

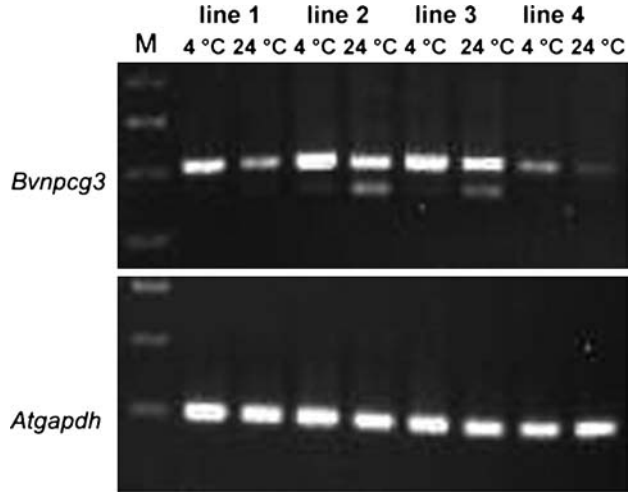

Fig. 4 Temperature regulated splicing of *Bvnpcg3* in *Arabidopsis thaliana*. RT-PCR analyses were performed with primers for *Bvnpcg3*, and the house keeping gene *Atgapdh* (Table 1) on cDNA generated from mRNA from four transgenic *Arabidopsis* lines (lines 1 through 4) that were either kept at 24°C or that were incubated at 4°C over night. The first lane (M) harbours a DNA size standard

was found that increased storage temperatures positively correlate with the amount of spliced product (Fig. 3c). For *Bvnpcg2* either equal amounts of both mRNAs or more of the third intron containing mRNA are amplified from leaves, non-stored and field clamp derived storage roots. Under controlled storage conditions at higher temperatures the intron-less mRNA increases compared to the intron-containing one. The different splicing efficiency of *Bvnpcg3* mRNA during storage is also prominent. In leaves, non-stored and field clamp derived storage roots, more of the intron containing mRNA than the intron-less mRNA is amplified (Fig. 3c). Under controlled storage conditions at higher temperatures the intron-less mRNA increases compared to the intron-containing one.

Temperature dependent splicing of the *Bvnpcg3* mRNA in *Arabidopsis thaliana*

To further investigate the temperature responsive splicing of the *Bvnpcg3* mRNA, the cDNA containing the second intron (Fig. 3a) was introduced into *Arabidopsis thaliana* under the control of the CaMV 35S promoter. Four independent transgenic lines were obtained and analysed by RT-PCR (Fig. 4). As a control, the transcript of the cytosolic GAPDH gene was amplified with primers that would also amplify an intron if this mRNA is unspliced. RT-PCR analysis was done on plants that were kept at 24°C and on plants that were incubated over night at 4°C. The control GAPDH gene could be amplified at 4°C and 24°C and no intron retention is observed because the presence of an intron in the PCR product would generate a larger fragment. In contrast, the *Bvnpcg3* transcript is mainly unspliced in all transgenic lines investigated. However, two transgenic lines increased splicing of the transcript at 24°C compared to 4°C (Fig. 4, lines 2, 3). This result demonstrates that higher temperatures stimulate splicing of the *Bvnpcg3* transcript also in *Arabidopsis thaliana*.

Discussion

To learn more about post-harvest specific gene expression, post-harvest upregulated genes were isolated by subtractive

cloning from sugar beet. Three of the 16 isolated genes do not encode a known protein and five genes have not previously been cloned. Two putative non-protein coding genes are post-transcriptionally regulated during storage. Both show intron specific splicing that positively correlates with increasing temperatures. Temperature regulated splicing of an intron containing sugar beet mRNA was also observed in transgenic *Arabidopsis thaliana*.

Based on the encoded proteins and the expression profile during storage a model can be discussed that proposes the response to several different post-harvest stress conditions in storage roots. The largest group of genes isolated are those that play a role in pathogen defense. Some of these genes belong to the classic families of pathogenesis related proteins such as *Bvcprx1*, *Bvglp-r* and *Bvsp2* (van Loon et al. 2006). The cationic peroxidase *Bvcprx1* is a homolog of members of the pathogenesis related protein family PR-9. The type member of this family is the lignin-forming peroxidase from *Nicotiana tabacum* (Lagrimini et al. 1987). This and BvCPRX1 show 36% amino acid sequence identity (data not shown). BvCPRX1 may play a role in the detoxification of active oxygen species (AOS). AOS are generated upon pathogen attack as defense substances and as signal transduction components (van Loon et al. 2006). Cationic peroxidases such as BvCPRX1 can degrade AOS in the so called peroxidative cycle (Passardi et al. 2005). An indirect role in the detoxification of AOS may be attributed to the cytochrome-b561 oxidase that is encoded by *Bvc561r* which could be involved in the redox control of the AOS scavenging antioxidant ascorbate (Asard et al. 2001). Cytochrome b561 oxidase is involved in regenerating ascorbic acid from monodehydroascorbate that is the product of ascorbic acid oxidation (Asard et al. 2001). *Bvglp-r* is a germin like protein similar to the PR-16 family and *Bvsp2* is a member of the PR-3 class of chitinases IV. The role of germin-like proteins is still not clear. They may have oxalate oxidase and superoxide dismutase activity or function as extracellular receptors (Bernier and Berna 2001). An extracellular localization of BvGLP-r is supported by the proposed amino terminal transit peptide. The acidic chitinase BvSP2 has been analysed before (Nielsen et al. 1994). Although transcript levels of *Bvsp2* increase after *Cercospora beticola* infection, BvSP2 has no antifungal activity against *Cercospora beticola* spores in vitro.

Other genes that are involved in pathogen defense are the two ribosome inactivating protein coding genes, *Bvrip-r1* and *Bvrip-r2*. Ribosome inactivating proteins were not assigned to a PR gene family because the proteins have not been reported to be induced upon pathogen attack (van Loon et al. 2006). However, recently it was shown that ribosome-inactivating proteins (RIPs) from sugar beet are induced during viral infection and may have a role in systemic aquired resistance (Iglesias et al. 2005). While *Bvrip-r2* is only transiently upregulated, *Bvrip-r1* is expressed at all time points and all storage conditions (Fig. 1). Ribosome-inactivating proteins (RIPs) are a group of N-glycosidases that specifically cleave nucleotide N-C glycosidic bonds (Park et al. 2004). The widely accepted mechanism for antimicrobial action identifies host ribosomes as the target of RIP activity while also DNase and RNase activity has been shown for RIPs. The signal peptides proposed for BvRIP-r1 and BvRIP-r2 point to a localization in either the apoplast (BvRIP-r2) or to a retention within the endoplasmatic reticulum (BvRIP-r1). This proposition is based on the cleavable signal peptide in BvRIP-r2 and the non-cleavable peptide in BvRIP-r1. The early induction of both proteins after harvest without experimental pathogen infection may point to a function for both proteins to prevent pathogen infection (Fig. 1).

Bvhsp83 encodes a heat shock protein of the HSP90 family. HSP-genes can be involved in the resistance gene mediated signal transduction pathway (Schulze-Lefert 2004). B12D proteins have a more elusive role in pathogen response (Aalen et al. 1994). They are upregulated during senescence but were also found to be upregulated in rice suspension cultures treated with a fungal elicitor (Huang et al. 2001).

Genes involved in pathogen defense overlap with those involved in wound response. During harvest the storage roots are wounded when the top of the beet is cut off and the beets are mechanically pulled from soil and transferred to clamps. Wound response reactions could involve the early expression of *Bvcprx1*, *Bvmarb*, *Bvrcc*, as well as *Bvpsk*. The cationic peroxidase BvCPRX1 could be responsible for catalysing cell wall lignification at the wound side (Passardi et al. 2005). The early expression of *Bvmarb* and *Bvrcc* could be an indicator for cell divisions involved in generating a wound periderm (Colon-Carmona et al. 1999). Regulator of chromatin condensation (RCC) plays a role in spindle fibre formation and the matrix attachment region binding protein (MARB) is required for chromosome condensation (Carazo-Salas et al. 1999; Fujimoto et al. 2004).

The upregulation of an aquaporin encoding gene, *Bvbpm1*, that responds to drought stress and whose encoded protein localizes to the plasma membrane may indicate a drought stress response during storage (Barone et al. 1997).

Genes that cannot be assigned to a specific function are a WD40 repeat encoding gene (*Bvwdrp*) and three genes that do not code for a known protein (*Bvnpcg1-3*). The function of these genes is puzzling and may only be speculated upon.

Non-protein coding genes are of increasing scientific interest because they may encode miRNAs, or may generate siRNAs or ta-siRNAs (Jones-Rhoades et al. 2006). Bioinformatic analysis of *Bvnpcg1, 2*, and *3* did not predict an encoded miRNA in any of the three non-protein coding

genes, except the unspliced mRNA of *Bvnpcg3*. Also, no homologous antisense transcript that may be required for the formation of siRNAs or ta-siRNAs has been identified in the databases. For *Bvnpcg3* a short stem loop structure that conforms to certain parameters required for a miRNA precurser could be proposed (data not shown). Interestingly, the short stem loop structure can only be proposed for the unspliced version of *Bvnpcg3* with its retained second intron. This was interesting because it may indicate splicing regulated miRNA formation. However, a *Bvnpcg3* corresponding miRNA could neither be detected in RNA gel-blots nor could one be cloned from storage roots (data not shown). Therefore, the functional relevance of the observed post-harvest increased splicing is not yet clear. Intron retention has been observed in plants for example in response to anaerobiosis and cold stress (Köhler et al. 1996; Mastrangelo et al. 2005). The observed intron retention of *Bvnpcg2* and *Bvnpcg3* is also promoted under low temperature conditions. Because higher temperatures positively correlate with increased splicing efficiency it may be speculated that temperature regulated splicing may be a factor to control gene expression during winter months and to rapidly sense increasing temperature during spring. If this mechanism is related to the vernalization process required for bolting in the second year remains to be analysed.

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